The levels of sulfur-containing metabolites in *Brassica napus* are not influenced by the circadian clock but diurnally

Johann Hornbacher[1,*] Annekathrin Rumlow[1,*] Philipp Pallmann[2] Ariel E. Turcios[1] Anja Riemenschneider[1] Jutta Papenbrock[1]

[1]*Institute of Botany, Leibniz University Hannover, Herrenhäuserstr. 2, D-30419 Hannover, Germany*

[2]*Centre for Trials Research, Cardiff University, Cardiff, CF14 4YS, United Kingdom*

*These authors contributed equally.

Corresponding author: Jutta Papenbrock
Leibniz University Hannover, Institute of Botany
Herrenhäuserstr. 2, D-30419 Hannover, Germany
Tel. 49 511 762 3788 Fax 49 511 762 19262
E-Mail: Jutta.Papenbrock@botanik.uni-hannover.de

Formatted: German (Germany)

Formatted: German (Germany)

Abstract Adapting biological processes to an endogenous rhythm enables plants to cope with the daily changes in light and temperature in a more predictable way enhancing growth and fitness. A number of biological processes such as metabolic pathways as well as the immunity in plants are under diurnal or circadian control. In this study a possible circadian regulation of key enzymes in the sulfur assimilation and the corresponding metabolites was investigated in the agriculturally important crop plant oilseed rape (*Brassica napus*). Leaves of a commercially available cultivar were harvested in the course of a day under diurnal and under free-running conditions with constant light. Analyses in this study were focused on sulfur-containing metabolites and expression analysis of enzymes involved in sulfur assimilation. Expression analysis showed that the transcript levels of the sulfate transporters *Sultr3;1* and *Sultr4;2* as well as *APR2* and *APR3* oscillated diurnally. Results revealed a periodic rhythm of sulfur-containing metabolites such as glutathione, sulfate and certain glucosinolates in the course of a day which were only partly maintained under constant light. Therefore, we conclude that a diurnal rhythm and not the circadian clock regulates sulfur metabolism in plants.

Keywords: *Brassica napus* Circadian clock Diurnal rhythm Expression analysis Sulfur-containing metabolites

Introduction

Life on earth is exposed to daily changes in light, temperature and other environmental factors due to the rotation of the earth. The metabolism, behavior and physiology in organisms were adapted to these daily changes, known as diurnal rhythms. In the absence of an external cue many of these oscillations persist and free-run with an endogenous period that is close to 24 h. These circadian rhythms are under the control of an endogenous circadian clock. Under natural conditions, light and temperature act as environmental timer entraining the endogenous organismic clock in each cell with the local time (McClung 2006; Harmer 2009).

The model of the circadian clock in plants has so far been best described in *Arabidopsis thaliana* (Salome and McClung 2004). The MYB domain transcription factors CIRCADIAN AND CLOCK ASSOCIATED 1 (CCA1) and LATE ELONGATED HYPOCOTYL 1 (LHY1) along with TIMING OF CAB 1 (TOC1) represent the core oscillator of the circadian clock as a negative feedback loop. Linked to this core oscillator further feedback loops are formed regulating the expression of so-called morning as well as evening genes (McClung 2006). Up to 80% of the transcriptome in rice (*Oryza* spp.), poplar (*Populus* spp.), and *Arabidopsis thaliana* are regulated by the circadian clock enabling the timing of a number of biological processes and stress responses, respectively (Filichkin et al. 2011). The photosynthesis as the primary biological process shows circadian oscillations in the light harvesting and a circadian-regulated CO_2 fixation by Rubisco (Dodd et al. 2014). The circadian rhythm is also involved in certain stress responses. The involvement of the circadian rhythm in reactive oxygen species (ROS) mediated stress was shown in *A. thaliana* where the overexpression of the *CCA1* led to an enhanced drought tolerance by increasing the expression of ROS controlling genes (Lai et al. 2012). However, the circadian rhythm is not solely involved in abiotic stress responses but also in plant immunity. Results from Wang et al. (2011) indicate that defense genes are under circadian control by CCA1, allowing plants to estimate infection at dawn when pathogens normally disperse the spores and time immune responses according to the perception of different pathogenic signals upon infection. The studies revealed a key functional link between the circadian clock and plant immunity. Mutants overexpressing *CCA1* showed enhanced resistance against downy mildew supporting a direct interaction of the clock with plant immunity (Wang et al. 2011).

The importance of the plant clock due to its role in agriculture is now rising (Shaw et al. 2012). As an agriculturally important oilseed crop *Brassica napus* is the most closely related species to the crucifer *A. thaliana* with a number of highly conserved genes among both species. Compared to other crops and cereals the requirements for nitrogen, phosphorus and sulfur are higher making it more sensitive to sulfur-deficient conditions (Schnug and Haneklaus 2005).

Sulfur is taken up by roots from the soil as inorganic sulfate. The uptake of sulfate by the roots and its transport to the shoot seem to be one major site of regulation of sulfur

assimilation. In *A. thaliana* and *B. napus* 14 sulfate transporter genes have been identified which are subdivided into five different groups with different affinities to sulfate and located in different organs and organelles (Hawkesford and De Kok 2006; Parmar et al. 2007). The function and localization of the sulfate transporters of group three was long unknown. A recent study revealed that the transporter *Sultr*3;1 is located in the chloroplast enabling the sulfate uptake of chloroplasts (Cao et al. 2013). Furthermore, transcriptome analysis revealed a circadian regulation of this transporter in *A. thaliana* (Covington et al. 2008). Members of group four are known to be localized at the tonoplast enabling the efflux of sulfate out of the vacuole. It was already shown that the transporter *Sultr4;2* in *B. napus* was only expressed under sulfur-deficient conditions, thus playing a major role in the response to sulfur deficiency (Parmar et al. 2007).

Feeding experiments using $^{35}SO_4^{2-}$ showed that the incorporation of ^{35}S into reduced sulfur compounds in vivo was significantly higher in light than in the dark (Kopriva et al. 1999) in accordance with investigations on adenosine 5'-phosphosulphate reductase (APR), considered to be a key enzyme of sulfate assimilation in higher plants. The mRNA levels of all three APR isoforms showed a diurnal rhythm, with a maximum at 2 h after onset of light. In summary, in higher plants *APR* mRNA, APR activity and *in vivo* sulfate reduction change with a diurnal rhythm, sulfate assimilation also takes place during the dark period, and sucrose feeding positively affects *APR* mRNA expression and APR activity in roots (Kopriva et al. 1999).

The first stable sulfur-containing compound in the sulfur assimilation cysteine acts, besides its role in the protein synthesis, as a precursor for essential biomolecules such as vitamins and cofactors. A small portion of the cysteine content is used for the biosynthesis of the tripeptide glutathione (GSH) (Hawkesford and De Kok 2006). Sulfur is also present in secondary compounds, i.e. glucosinolates (GSLs). GSLs play an important role in the response to biotic stress, especially in the defense against herbivores (Mithöfer and Boland 2012). In recent years their role in abiotic stresses such as salinity, drought, extreme temperatures, light cycling, and nutritional deficiency have been discussed (Boestfleisch et al. 2017). Previous studies revealed a circadian regulation of genes involved in the biosynthesis of GSLs (Kerwin et al. 2011).

There is emerging evidence for a relationship between the nutrient status and circadian rhythm in plants (Haydon et al. 2015). However, a direct interaction of the circadian clock and the sulfur status was not analyzed in detail so far. A number of key genes in metabolic pathways including the sulfur metabolism have been reported as circadian-regulated in *A. thaliana* (Harmer et al. 2000). In order to analyze the effect of sulfur availability on the circadian rhythm, two sulfur concentrations were chosen based on previous studies. Sulfur sufficient control plants received 1 mM $MgSO_4$ based on the studies of Blake-Kalff et al. (1998). Previous experiments revealed 10 µmol $MgSO_4$ to be ideal to study the effect of sulfur deficiency in plants by meeting their minimal sulfur needs and keeping plants alive (Weese et al., 2015).

Formatted: Subscript
Formatted: Font: 13 pt
Formatted: Font: 13 pt
Formatted: Font: 13 pt
Formatted: Subscript

So far, diurnal and circadian changes in sulfur metabolism have only been described in *A. thaliana*, lacking essential insight into important crop plants like *B. napus*. Understanding the pattern in which sulfur is assimilated and sulfur-containing metabolites are synthesized is essential to be able to optimize sulfur fertilization, which in turn would maximize crop resistance to biotic stressors and tolerance to abiotic changes.

~~To obtain more insight into the circadian regulation of sulfur metabolism a current commercially available cultivar of *B. napus* was chosen and cultivated on sand, 1) to be able to generate a different sulfur status by watering with nutrient solutions containing two different sulfate concentrations and 2) to obtain a similar phenotype as during cultivation on soil. Plants were entrained to a 12 h light/12 h dark cycle and harvested during the course of a day. For analyzing gene expression and metabolites under free-running conditions, plants were exposed to continuous light. As the sulfur status might lead to alterations in the clock period, plants were additionally grown under sulfur-deficient conditions for 4 d. Sulfur-containing metabolites and selected genes as part of the sulfur assimilation pathway as well as sulfate transporters were analyzed under diurnal and circadian conditions.~~

Formatted: Font: Italic

Formatted: Font: Italic

Formatted: Font: Italic

Results

Establishment of a highly controlled cultivation system and investigation of the role of sulfur in a circadian-regulated system by expression analysis

For investigating the interaction of the circadian clock and sulfur metabolism a suitable cultivation system had to be established. Clock-controlled genes as well as genes involved in sulfate transport and assimilation were analyzed by Northern blot analysis (Fig. 1, Fig. S1). For analyzing the expression data in a quantitative way, results of Northern blot analysis were normalized according to Rumlow et al. (2016) with a validated set of reference genes (Fig. 1A, Table 1). It was demonstrated before that whereas RT-qPCR is currently the most used method to perform gene expression analysis, it was shown that similar results can be obtained using Northern Blot analysis. However, a set of suitable reference is essential to guarantee a confident evaluation of gene expression analysis (Rumlow et al. 2016). To check the stability of the cultivation system, the expression of *B. napus CCA1* gene was analyzed (Fig. 1A). Its expression pattern in light/dark (LD) conditions showed a maximum of expression in the light phase and remained undetectable at night. Regarding the sulfur fertilization, data revealed a statistically significant difference (p<0.0001) in the transcript level of *CCA1*, with the highest value under sulfur-deficient conditions (Table S1). In samples from plants harvested under light/light (LL) conditions the expression pattern remained the same. Moreover, the interaction effect between the three factors is statistically significant

(p<0.0001) (Table S1). Consequently, the transcript level of the variable *CCA1* may depend on the sulfur status, time point of harvest as well as the light conditions.

To be able to follow the sulfur status in the plants the sulfate transporter *Sultr4;2*, was included into the investigation. Plants grown in our experimental system showed a significant up-regulation of *Sultr4;2* when supplied with 0.01 mM $MgSO_4$ indicating a successful application of sulfur limitation (Fig. 1A). However, the transcript levels fluctuated in the course of a day with the highest transcript levels in the middle of the light phase. The expression of *Sultr4;2* was not significantly influenced by light condition. Furthermore, the influence of the sulfur status on the expression seems independent from the light (Table S1).

Northern blot analysis of *Sultr3;1* in plants grown under sufficient sulfur supply showed a notable up-regulation under LD conditions for approximately 8 h beginning 1 h before the onset of light. Followed by a considerable decrease in the transcript level, transcript amounts were maintained at a lower level in the dark phase and were increased again at 24 h (Fig. 1A). At 28 h transcript amounts of *Sultr3;1* were 2-fold higher compared to the measured transcript amount at 4 h. Regarding the sulfur status there was no significant influence on the expression of *Sultr3;1* measureable. A p-value of 0.6414 was calculated for the factor sulfur supporting the independence of the *Sultr3;1* expression from the sulfur status. In LL conditions analysis of the transcript levels resulted in a significant decrease in the subjective night compared to LD conditions. Furthermore, the transcript maximum of *Sultr3;1* was detected 8 h later than in plants grown under LD conditions. There is a highly significant interaction of light and the harvesting time point independent from the sulfur status (Table S1).

Furthermore, the expression of members of the *APR* gene family was analyzed (Fig. 1A). The expression of the *APR2* gene oscillated for both sulfur regimes in plants harvested under LD conditions during the course of a day with an up-regulated expression in the light phase. In plants grown under sulfur-deficient conditions transcript amounts of *APR2* were considerably higher in the light phase compared to plants grown under sufficient sulfur supply. The highest transcript amounts were measured in the beginning and in the middle of the light phase, respectively. In plants harvested under LL conditions the oscillations detected in the transcript levels of *APR2* were significantly affected by the light dependent on the time point the plants were harvested (Table S1). For the second isoform *APR3* expression analysis in the plants harvested under LD conditions resulted in nearly the same oscillations when compared to *APR2.* Sulfur-deficient conditions led to a significant increase in the transcript amount measured in the plants (Table S1). The oscillations in the transcript level of *APR3* in sulfur-deficient plants was much more pronounced compared to those grown under sufficient sulfur supply. Analyzing the expression of *APR3* in plants harvested under LL conditions resulted in a shift in the expression pattern which was highly dependent on the sulfur status. Only in plants grown under sulfur-deficient conditions the up-regulation began 4 h earlier in LL

conditions compared to the expression in LD conditions. Comparing both isoforms, the expression of *APR3* was more influenced by the LL conditions.

As all transcripts oscillated diurnally with an upregulation in the light phase, the wavelength of the rhythmic oscillation was determined using AICc selection based on a set of candidate models with trigonometric functions representing different wave lengths (see Statistical analysis for more information) for each GOI, respectively (Fig. 1B). Oscillations for the isoform *APR2* showed a 23 h rhythm under LD as well as under LL conditions, whereas for the latter one a lowered amplitude and an advanced phase (shifts earlier in time) was shown. For *APR3* oscillations comprise only a 20 h period and the amplitude under sulfur-deficient conditions was increased. Under sulfur-deficient conditions an advanced phase was shown. For the clock gene *CCA1* periodic oscillations of 23 h were determined. The amplitudes in the oscillations were unaffected by the sulfur status. Under LL conditions a delayed phase (shifts later in time) was shown. For the sulfate transporter *Sultr3;1* oscillations in the transcript level follow a 23 h rhythm. The amplitude was unaffected by the sulfur status but lowered under LL conditions and showed a delayed phase of 4 h. For the second transporter *Sultr4;2* the model of a 20 h rhythm was determined. In plants under sufficient sulfur supply the amplitudes of the oscillations were very low compared to the amplitudes of the oscillations under sulfur-deficient conditions. As the expression was unaffected by light no differences in the amplitude between LD and LL conditions were observed.

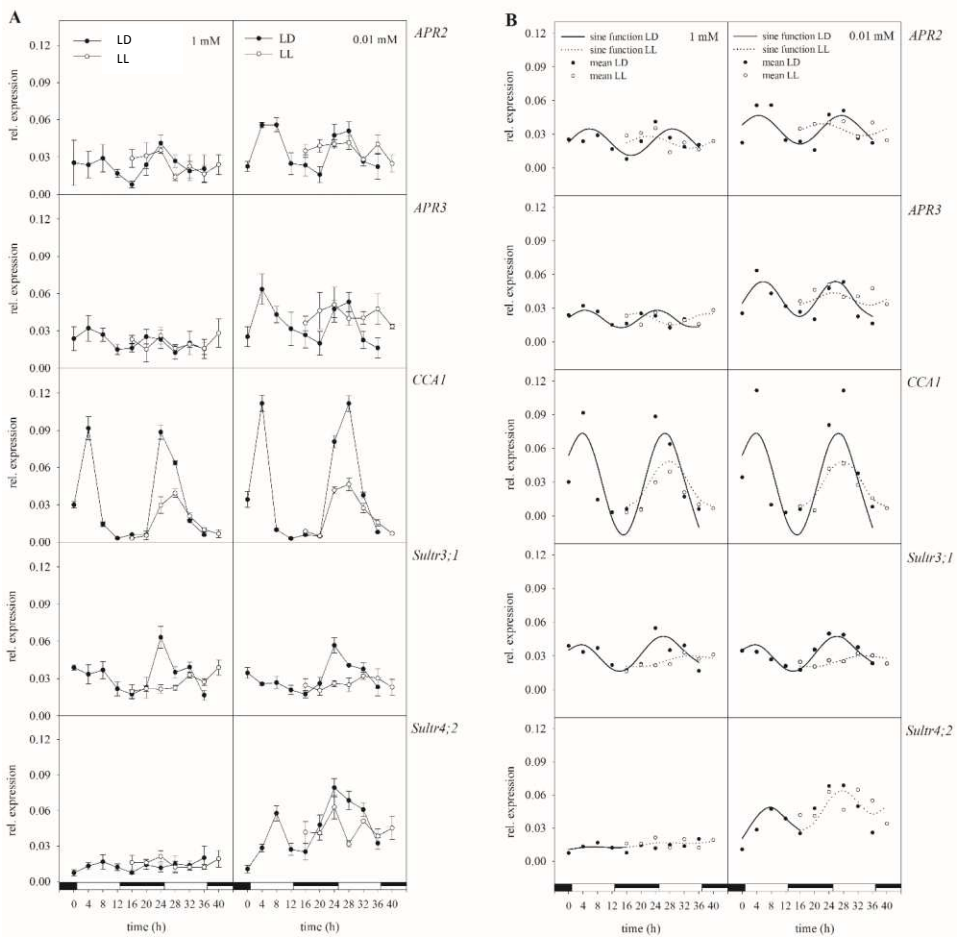

Fig. 1 Expression of GOIs under circadian and sulfur-deficient conditions. Transcript amounts were determined in above ground plant material of plants (with five fully expanded leaves) grown using 1 mM $MgSO_4$ (+S) as a control and 0.01 mM $MgSO_4$ (-S) for four days to obtain S-deficient conditions. Plants grown under 12 h dark/ 12 h light (LD, closed circles) were harvested over a period of 36 h every 4 h starting 1 h before the onset of light. In addition, plants grown in a chamber with 24 h light (LL, open circles) were parallel harvested beginning at 16 h and ending at 40 h fulfilling a 24 rhythm. Three plants per treatment and harvest time point were pooled. Total RNA was isolated, and for Northern blot analysis 15 µg RNA was electrophoretically separated and transferred onto membranes. For the detection DIG labeled probes were used. A) Normalization of the GOIs with a validated set of reference genes under LD and LL conditions as described and documented in detail in Rumlow et al. (2016). Data are shown as the mean of three technical replicates ± SD. Relative expression calculation was based on band intensity. B) Sine functions of the oscillations for the GOIs

together with the according mean from the three technical replicates under LD and LL conditions. Abbreviations for probes see Table 1.

Metabolic analysis of sulfur-containing compounds in a circadian-regulated system

Measurements of total sulfur amounts in leaves

Previous results indicated changes of the total sulfur content during the day (Weese et al., 2015). To understand the influence of the light period on the total sulfur content in the leaves, dried material was analyzed by ICP-OES. In addition, the effect of the sulfur treatments could be followed. The total sulfur content in plants grown under 1 mM sulfur supply and LD conditions showed slightly varying amounts during light and dark phases (Fig. 2, Table S1). In plant material harvested from plants grown under sulfur-deficient conditions the sulfur content was significantly decreased reaching 2.7 mg g^{-1} DM at 36 h (Table S1). Furthermore, the measured content was maintained at relatively constant levels in the plants harvested under LD conditions. Under LL conditions the sulfur content measured in the plants decreased irrespective of the sulfur status (Table S1). In the plant material from plants grown under sufficient sulfur supply and harvested under LL conditions the sulfur content decreased by up to 4.4 mg g^{-1} DM in the course of the day. For both sulfur regimes and light conditions variations in the content did evidently not follow a rhythmic pattern over time.

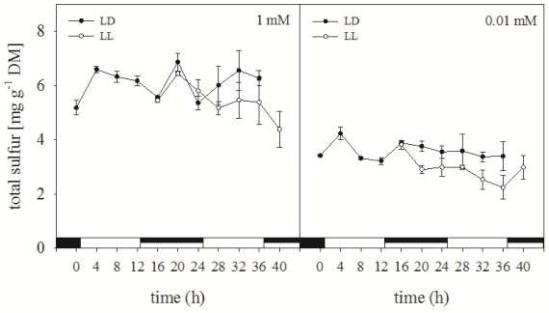

Fig. 2 Total sulfur under circadian and diurnal conditions. The elemental sulfur was measured in dried above ground plant material (DM) of plants treated as described in Figure 1 with ICP-OES. Results calculated as mg g^{-1} DM represent the mean of only two technical replicates ±SD due to limited amount of plant material.

Determination of sulfate levels in the leaves of *B. napus* plants

As the expression of the sulfate transporters *Sultr3;1* and *Sultr4;2* showed oscillations in the course of a day (Fig. 1), the sulfate contents measured in plants grown with sufficient

sulfur supply oscillated with maxima of approximately 5 mg g^{-1} DM 1 h before the onset of light and 1 h before the offset of light (Fig. S2, Table S1). Lowest amounts of sulfate were measured in plants harvested in the dark phase with 3.5 mg g^{-1} DM. In plants grown under sulfur-deficient conditions the measured sulfate content decreased in the light phase from 3.6 to 2.1 mg g^{-1} DM. After an increased content measured in the plants harvested in the dark phase the content of sulfate was further decreased down to 1.3 mg g^{-1}. For both sulfur regimes the measured sulfate amounts in the plants were significantly decreased under LL conditions which was highly dependent on the sulfate status and the time point of harvest (*p*-value <0.0001), respectively (Table S1). With a decrease of 60% of sulfur in plants grown under sufficient sulfur in LL conditions the decrease was more drastic compared to plants grown under sulfur deficient conditions. In conclusion, the interaction of all three factors influencing the sulfate content in the plants was highly significant (*p*-value 0.0001, Table S1).

Cysteine and glutathione contents in leaves

Due to its importance as key molecule in the primary sulfur assimilation pathway the cysteine content and the GSH content as the most important transport molecule of reduced sulfur were analyzed via HPLC (Fig. 3). The cysteine concentrations measured in plants grown with 1 mM $MgSO_4$ and harvested under LD conditions were higher during the day with a maximum of approximately 20.6 nmol g^{-1} FM than at night with a minimum of about 11 nmol g^{-1} FM (Fig 3A). Under sulfur-deficient conditions measurements of cysteine resulted in significantly lower contents with a minimum of approximately 9.9 nmol g^{-1} FM without any discernable oscillations in the course of a day. The content of reduced GSH measured in plants grown with 1 mM $MgSO_4$ oscillated diurnally in the course of a day with high amounts up to 560 nmol g^{-1} FM at the end of the light phase and significantly lower amounts of 240 nmol g^{-1} at the end of the dark phase (Fig. 3B). The decrease of the GSH measured in plants grown under sulfur-deficient conditions was highly significant with a *p*-value of 0.0002 (Table S1). With a maximum of approximately 403 nmol g^{-1} FM in the light phase and a minimum of 269 nmol g^{-1} FM the measured contents oscillated as well in the course of a day. Regarding the third factor “light” the GSH content measured in the plants harvested under LL conditions was significantly influenced dependent on the time points the plants were harvested. In agreement with the diurnal oscillations of the GSH content periodic oscillations with a period of 23 h was determined (Fig. 3C). The amplitude in LL conditions was lowered for both sulfur regimes. Under sulfur-deficient conditions an advanced phase could be observed. To summarize the results for the thiols, cysteine was only affected by the sulfur status and showed no oscillations in its content. The GSH content in contrast was influenced by all three factors. The factor that influenced the oscillations of the GSH content most was the time point the plants were harvested.

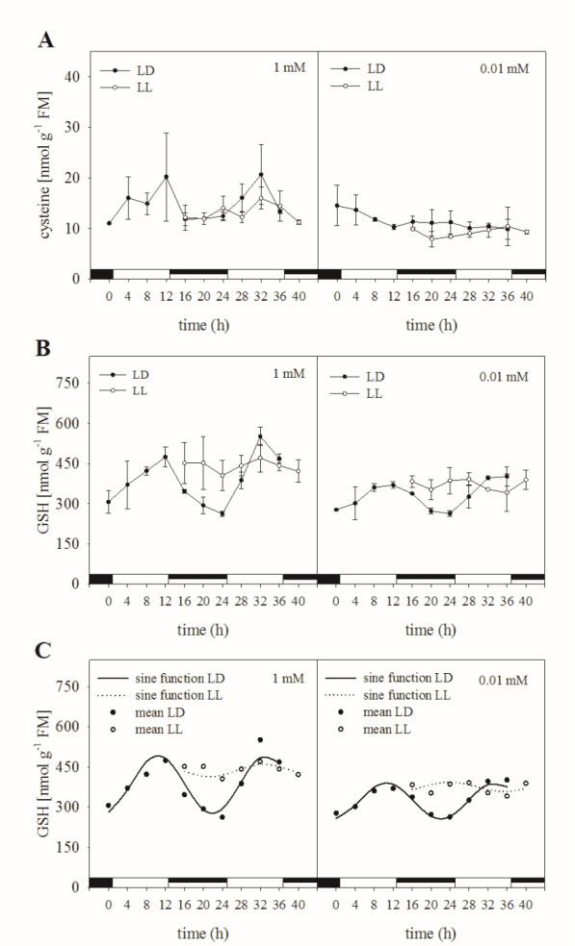

Fig. 3 Thiol contents under circadian and diurnal conditions. Cysteine (A), GSH (B), the sine function of the periodic oscillations with the corresponding mean (C). The cysteine and GSH contents were determined in above ground plant material of plants treated and collected as described in Figure 2 by HPLC. Data in nmol g^{-1} FM represent the mean of three technical replicates ±SD.

Quantification of GSLs in leaves

As representatives of the secondary sulfur metabolism intact GSLs were measured as desulfated GSLs in the leaves (Fig. 4, Fig. S3, S4). Taking all aliphatic GSLs together the measured content in plants grown with sufficient sulfur supply and harvested under LD conditions was maintained from 0 to 12 h at approximately 0.7 µmol g^{-1} DM (Fig. 4A). This was followed by a substantial increase in the content of aliphatic GSLs up to 1 µmol g^{-1} DM in the beginning of the dark phase (Table S1). After a slight decrease in the content in plants harvested later in the dark phase the content reached 1 µmol g^{-1} DM again 1 h after the onset of light. Afterwards the measured content of the aliphatic GSLs in the plants was decreased by 20% at 32 h and increased up to 0.9 µmol g^{-1} DM again at 36 h. The content of the aliphatic GSLs was significantly decreased in plants grown under sulfur-deficient conditions (Table S1) and the pattern in the oscillations in the content was slightly shifted, but more pronounced. Considering the third factor light the content of the aliphatic GSLs was significantly influenced independent of the sulfur status (Table S1). Under sufficient sulfur supply the amount of aliphatic GSLs measured in plants harvested under LL conditions was lower in the subjective night at 16 and 20 h compared to plants harvested under LD conditions. In plants grown under sulfur-deficient supply and harvested under LL conditions the content of the aliphatic GSLs was significantly lower compared to plants harvested under LD conditions without any oscillations (Table S1). Regarding the individual aliphatic GSLs nearly the same pattern in the oscillations could be observed except for glucoraphanin (Figure S3). The oscillations in the content of the individual aliphatic GSLs were highly dependent on the sulfur status (*p*-value <0.0001) (Table S1). Interestingly gluconapin and glucoraphanin were not significantly influenced by the light independent from the sulfur status and the time point the plants were harvested.

In plants grown under sufficient sulfur supply and harvested in LD conditions, the highest concentration of the indolic GSLs with 0.15 µmol g^{-1} DM was measured at 0 h and 12 h respectively (Fig. 4B). Afterwards the content was decreased down to approximately 0.1 µmol g^{-1} DM and maintained at this level. Although the content of the indolic GSLs was not considerably decreased in plants grown under sulfur-deficient conditions an altered pattern in the oscillations was observed (Fig. 4B). A maximum of approximately 0.25 µmol g^{-1} DM was measured in plants harvested three hours after the onset of light. Afterwards the content was decreased by about 70% in plants harvested at 8 h. In the beginning of the dark phase the content of the indolic GSLs was increased again to 0.15 µmol g^{-1} and decreased to 0.1 µmol g^{-1} DM at the end of the dark phase. In plants harvested in LL conditions the content of the indolic GSLs was not influenced in a considerable way independent of the sulfur status and the time point the plants were harvested (Table S1). Comparing the two GSLs glucobrassicin and neoglucobrassicin representing the indolic GSLs, for the former one oscillations were higher in the course of the day (Fig. S4).

The only aromatic GSL measured in *B. napus* was gluconasturtiin (Fig. 4C). In plants grown with sufficient sulfur supply and harvested under LD conditions a content of

approximately 0.06 µmol g^{-1} DM was measured one hour before the onset of light. The decrease in the content down to 0.05 µmol g^{-1} DM measured in plants harvested at 4 and 8 h was followed by a notable increase up to 0.085 µmol g^{-1} DM four hours later. In the middle of the night phase the content of gluconasturtiin was decreased by 50%. Already 4 h later the measured content was increased again up to 0.065 µmol g^{-1} DM. At the end of the light phase the content in the plants was decreased by 30%. The sulfur-deficient conditions led to no significant decrease in the content of the aromatic GSL, whereas the pattern of the oscillations in the content in plants harvested under LD conditions was altered with higher contents at 4h and 20 h. The oscillations of gluconasturtiin in both sulfur and light regimes is very similar to the oscillations of indolic GSLs. Regarding the third factor light the content of gluconasturtiin in the plants was affected in a significant way independent from the sulfur status (Table S1). In plants grown with sufficient sulfur supply and harvested under LL conditions the content was decreased by 50% between 24 and 40 h. In plants grown under sulfur-deficient conditions the content of gluconasturtiin was much lower compared to plants harvested under LD dark conditions (Table S1). Interestingly, all GSL groups do not show clear oscillations when supplied with sufficient sulfur, whereas under sulfur deficient conditions oscillations in the content occurs in all GSL groups with the indolic and aromatic ones showing more pronounced amplitudes. For the indolic and aromatic GSLs the amplitude of the oscillation in sulfur deficient plants is the highest at the first morning in the harvest period getting smaller over time. The frequency of the oscillation remains the same over the harvest period. To summarize the measurements of the GSLs in *B. napus* the oscillations in the content of the GSLs were dependent on the sulfur status. Only the aliphatic GSLs were reduced in their content under sulfur-deficient conditions. Furthermore, only for the indolic GSLs the content was unaffected by the circadian conditions.

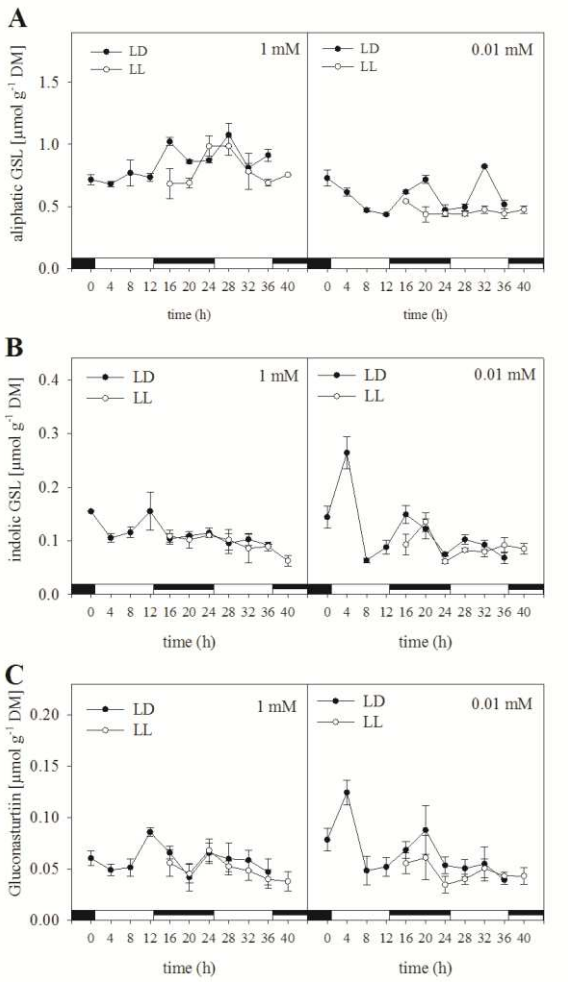

Fig. 4 GSLs in leaves under diurnal/circadian conditions. Aliphatic (A) and indolic GSLs (B) as well as one aromatic GSL (C) were measured by HPLC in above ground plant material of plants treated and collected as described in Figure 2. The contents of the GSLs were calculated in µmol g^{-1} FM. Data represent the mean of three technical replicates ±SD.

Discussion

Investigating circadian aspects of sulfur metabolism in *B. napus* in a reproducible way by establishing a suitable growth system is possible

For analyzing the influence of the circadian clock on the biosynthesis of sulfur-containing metabolites a highly controlled growth system had to be established. By analyzing the expression of the clock gene *CCA1* as a regulatory element of the core oscillator of the circadian clock stability of the cultivation system was verified. The expression was analyzed in plants collected every 4 h over a period of 40 h under diurnal conditions with 12 h light and 12 h dark as well as under free-running conditions with 24 h continuous light, respectively (Fig. 2, Fig S1). The expression pattern from Northern blot analysis of *CCA1* in *B. napus* is switched to early expression with a peak at ZT4, probably due to the 12 h light/ 12 h dark rhythm but the overall pattern is comparable to the pattern in other plant species (Mizoguchi et al. 2002; Harmer 2009) indicating a suitable cultivation and harvest pattern. The amplitude under constant light was significantly lowered similar to results obtained by transcriptome analysis of *CCA1* in *A. thaliana* (Mockler et al. 2007). In the study of Kim et al. (2003) it was discussed that light may influence the activity of a positive effector of *CCA1* and *LHY* leading to an altered amplitude of the transcript level. As a possible candidate they named the TOC1 protein as it showed interactions with a phytochrome B-related transcription factor protein. On the basis of the expression analysis it was possible to verify a circadian period of 23 h for *CCA1* in *B. napus.* Comparable to this study the "zeitgeber" was light, a circadian period of 24 h would be assumed. It was already demonstrated in *A. thaliana* and *Brassica rapa* that shortening of the period is dependent on the temperature (Kusakina et al. 2014). Plants grown under 17°C showed a circadian period around 24 h whereas under 27°C the period was shortened down to 20 h. It is postulated that a shorter period at higher temperatures may confer a performance advantage. Therefore, it can be suggested that the circadian period of the clock with 23 h in *B. napus* is more beneficial when grown at a temperature of 22°C.

Furthermore, half of the plants were grown under sulfur-deficient conditions for 4 d. This was done to determine a possible influence of sulfur limitation on the circadian clock in the first place. There are indeed a number of nutrients which are influenced by the circadian rhythm and vice versa (Haydon et al. 2015). The nitrogen metabolism in *A. thaliana* is regulated by *CCA1* which binds to the promoters of nitrogen-assimilation genes (Gutiérrez et al. 2008). In our study this was not the case as the period of *CCA1* was unaffected by sulfur-deficient conditions. Therefore, it can be concluded that in our study the given conditions have no influence on the expression of clock transcripts. Therefore, to verify the sulfur deficiency in the plants the expression of the sulfate transporter *Sultr4;2* was analyzed (Fig. 2) which is predominantly detectable under sulfur-deficient conditions (Buchner et al. 2004). In plants grown under sulfur-sufficient

conditions low amounts of transcripts were detected. One explanation would be that the supply of 1 mM $MgSO_4$ was not enough for a fully sufficient supply. Another explanation would be that a high degree of excessive sulfate triggered the up-regulation of the transporter. Thus, the efflux of the sulfate out of the vacuole is triggered preventing an over-accumulation of sulfate in the vacuole (Kataoka et al. 2004; Reich et al. 2017). In plants grown with 0.01 mM $MgSO_4$ the expression of *Sultr 4;2* was up-regulated. This was also confirmed in previous studies with *B. napus* where this transporter was up-regulated under sulfur-deficient conditions (Buchner et al. 2004; Parmar et al. 2007; Weese et al. 2015).

The sulfate transport is differently affected by the diurnal and circadian conditions

In previous studies the expression of all members of the sulfate transporters in the four groups in *Brassica oleracea* and all members of the five groups in *B. napus* under sulfur deprivation were analyzed in detail (Buchner et al. 2004; Parmar et al. 2007). In *B. oleracea* the sulfate transporter *Sutlr3;1* was expressed in the stem and roots independent from the sulfur supply of the plants. However, in leaves the expression of this transporter was only up-regulated under sulfur deprivation lasting at least 10 d (Buchner et al. 2004). Interestingly, in the study by Parmar et al. (2007) the expression of the transporter *Sultr3;1* could not be detected at all in the leaves of *B. napus* plants. This is in contrast to our results as the transporter was expressed at a relatively high level independent from the sulfur status (Fig. 2). Furthermore, transcript levels oscillated under diurnal and circadian conditions with a period of 23 h whereas under free-running conditions the amplitude was decreased and delayed in the phase. As the period matches the period of *CCA1* and remained the same in LL conditions a regulation by the circadian clock can be assumed. This is even further supported as in *A. thaliana* the transporter *Sultr3;1* contains an evening element promoter motif (EE) (http://arabidopsis.med.ohio-state.edu/) where CCA1 can directly bind and regulate the expression (Harmer et al. 2000; Nagel et al. 2015). Genes with this motif are likely to be expressed in the evening as binding of CCA1 in this promoter region enhances the expression. Therefore, one would expect a down-regulation in the expression of *Sultr3;1* in the morning and an up-regulation in the evening. This is contradictory to our results, as the expression was up-regulated in the morning and down-regulated in the evening (Fig. 2A, B). However, our results for the expression of *Sultr3;1* are in agreement with microarray analysis in *A. thaliana* under different light conditions (Mockler et al. 2007). In the study by Nagel et al. (2015) a number of target sequences for CCA1 close to the EE were genome-wide identified in *A. thaliana* revealing a morning-phased expression. Based on that it can be assumed that the sequence of the *Sultr3;1* in *B. napus* contains no EE and instead one of the other targets, thus leading to a peak of the expression in the beginning of the light phase. This is confirmed by our results as the transcript levels peak in the beginning of the light phase when *CCA1* was up-regulated as well. Confirming

a regulation by the circadian clock one would assume that under continuous light oscillations of the transcript levels of *Sultr3;1* would be unaffected. However, in this study the amplitude was lowered and a delay in the phase occurred. A possible explanation could be the transcript amount of *CCA1* which was significantly decreased under free-running conditions. Thus, the amplitude in the beginning of the light phase for the transcript level of *Sultr3;1* was decreased as well. Nonetheless there is evidence that sulfur uptake in chloroplasts is regulated by the clock due to the circadian-regulated sulfate transporter 3;1 which is localized at the chloroplast membrane (Haydoen et al. 2011; Cao et al. 2013). It is postulated that other members of group 3 transporters are also located at the chloroplast membrane (Cao et al. 2013). Therefore, expression analysis of the other members would be helpful for confirming a circadian-regulated uptake into the chloroplast.
The expression of *Sultr4;2* was predominantly analyzed as detection for the sulfur limitation (Fig. 2). Diurnal oscillations of the transcript levels under sulfur-deficient conditions were detected and were unaffected under free-running conditions in the subjective night. Based on that one could assume a regulation by the circadian clock. However, as the expression of the group 4 transporters is probably solely regulated by a sulfate gradient at the tonoplast a direct circadian regulation is likely to be ruled out (Kataoka et al. 2004; Reich et al. 2017).

The isoforms of the key enzyme in the sulfate reduction are affected differently in *B. napus* under diurnal and circadian conditions

As key enzymes of the sulfate assimilation pathway the expression of two isoforms of the adenosine-5'-phosphosulfate (APS) reductase (APR) was analyzed (Fig. 2). According to sequence analysis by using BLAST all three isoforms present in *A. thaliana* are also present in *B. napus.* As in the study by Kopriva et al. (1999) *APR1* and *APR3* showed a similar expression, for our study only the isoform *APR3* was included in the expression analysis in addition to *APR2*. For both isoforms transcript amounts oscillated diurnally with higher transcript amounts in the light phase under sulfur-sufficient as well as under sulfur-deficient conditions. This is in accordance with the expression of *APR* in *A. thaliana* and maize, oscillating diurnally with a maximum during the light period (Kopriva et al. 1999). Under sulfur-deficient conditions the amplitude of the oscillations increased. The periodic oscillations in the transcript level of *APR2* comprise 23 h with a peak in the morning phase which is equal to the period of *CCA1*, thus supporting a regulation by the circadian clock. Under free-running conditions oscillations of 23 h were observed whereas the amplitude was lowered and showed an advanced phase with a peak in the subjective night. In the case of *APR3* oscillations last only 20 h. The amplitude was lowered by the continuous light and an advanced phase appeared under sufficient sulfur supply. In the study of Kopriva et al. (1999) the regulation of *APR* expression and APR activity by an endogenous rhythm was ruled out as mRNA levels of

all three isoforms decreased in continuous dark. However, it was already reported that the expression of *APR2* is under circadian control in *A. thaliana* (Harmer et al. 2000) which is in accordance with the presence of the EE in the sequence of *APR2* (http://arabidopsis.med.ohio-state.edu/). As already described for *Sultr3;1* genes with an EE are likely to peak in the night. This is in agreement with the results from the microarray analysis in *A. thaliana* as highest transcript amounts were measured in the night and lowest in the day, respectively, independent of the given light conditions (Mockler et al. 2007). As *APR2* transcripts contribute 75% of the APR activity in *A. thaliana* one could assume that the APR activity would show the same oscillations. However, in previous studies APR activity had only been shown to undergo a diurnal rhythm in plants adapted to short days; when plants were grown in long days APR activity was again higher during the light period than in the dark, but without the strong maximum observed under short days (Huseby et al. 2013). It remains remarkable that the expression of *APR2*, *APR3*, and *Sultr3;1* under LD conditions has been drastically delayed in LL conditions. In summary, the results obtained in our study are contradictory for *APR2* regarding the circadian regulation thus making a proper conclusion difficult. Future experiments will be necessary to better understand the regulation of the expression of all gene isoforms involved in sulfur metabolism.

Is there a circadian regulation in the transport and reduction of sulfate?

In this study the aim was to determine whether the transport and the reduction of sulfate might be under circadian control. For investigating an influence by the circadian clock, the light was chosen as "zeitgeber". Plants grown with sufficient sulfur supply as well as with sulfur-deficient supply were entrained to a 12 h light/dark rhythm. By exposing part of the plants to continuous light the external cue was absent. Under these conditions circadian regulated genes should show the same oscillations as under diurnal conditions. Except for *Sultr4;2* oscillations in the transcript levels of the analyzed GOIs showed lowered amplitudes and in some cases an advanced or delayed phase, whereas the period of the oscillations under these conditions remained the same. (Fig. 2B). Nevertheless, a circadian regulation could not be unambiguously determined. It can only be assumed that there might be a regulation by the circadian clock as the transporter *Sultr3;1* and *APR2* oscillate in the same period as *CCA1* with a peak in the morning under diurnal conditions. Therefore, a direct interaction of CCA1 with the target genes by binding to specific binding motives might be possible (Nagel et al. 2015).
However, under LL conditions there was a shift in the phase and a lowered amplitude by up- or down-regulation even though the period of the oscillations remained the same. Potential targets of CCA1 were identified genome-wide in *A. thaliana* by ChipSeq analysis under LD and LL conditions (Nagel et al. 2015). A large portion of the putative target genes were non-cycling under LL conditions. It was discussed that this might be stress-related as plants were not expecting light in the night and consequently did not

cycle in LL conditions (Velez-Ramilez et al. 2011). As a response to the LL induced stress the generation of reactive oxygen species (ROS) might be triggered. As GSH is involved in the detoxification of ROS (Noctor et al. 1997) it is likely that under LL conditions GSH accumulates in the plants thus leading to an altered activity of the enzymes involved in the sulfur assimilation as here the precursor for GSH cysteine is formed. It was shown in *A. thaliana* that the sulfate transport is negatively regulated by GSH thus leading to a decrease in the expression (Vauclare et al. 2002) which would be an explanation for the down-regulation of *Sultr3;1* under LL conditions. Based on that a possible regulation of the sulfur assimilation by the clock might be underestimated by the use of LL. As the sulfur assimilation is dependent on the reducing equivalents produced in the photosynthesis, which is circadian regulated, a regulation by the circadian clock especially for the transport into the chloroplast is plausible (Harmer et al. 2000).
Measuring the total sulfur content in addition to the detection of the sulfate transporter *Sultr4;2* was a second way to detect sulfur deficiency in plants. *Brassica napus* plants with a sulfur content of 3.5 mg sulfur g^{-1} DM are considered to suffer deficiency (Scherer 2001). This is in accordance with the data obtained in our study (Fig. 3). Following the total sulfur content in the course of a day, can give first information on the regulation of the sulfur metabolism. The total sulfur content showed variations in the course of the day, however, statistical analysis showed diurnal oscillations are unlikely. As sulfate-sulfur comprises the biggest portion of the total sulfur in plants (Blake-Kalff et al. 1998) it was not surprising that the content did not oscillate diurnally (Fig. S2). Interestingly, under constant light the sulfate-sulfur content and consequently the total sulfur showed a decrease. The lower contents of sulfate might be due to a stress response induced by the LL as mentioned before resulting in an increased use for the generation of GSH. In the period of 40 h the plants were harvested, sulfate and consequently total sulfur levels decreased which might be due to decreasing sulfate amounts in the sand the plants were grown in. It would have been necessary to measure the sulfate content and its decrease in the substrate which might have led to the decrease of sulfur in the plants.

Glutathione shows diurnal oscillations

In previous experiments it was reported that the major sulfur-containing metabolite GSH, as representative of primary sulfur metabolism showed only minor or no fluctuation during a light/dark period. GSH levels were higher during the light period than in the dark, without a clear maximum. On the other hand, no diurnal changes in cysteine or GSH contents were observed in poplar (Noctor et al. 1997). In our study, GSH showed diurnal oscillations with a period of 23 h with a maximum at the end of the light phase (Fig. 3B, C). Same oscillations in the content of GSH could be observed in plants harvested in LL conditions, whereas the amplitude of the oscillations was lowered and a delay in the phase under sulfur-deficient conditions occurred. As mentioned before, this might be based on the continuous light plants were exposed to, as well as the stress

caused by sulfur deficiency. As a consequence, ROS might have been accumulated which can oxidize GSH to GSSG and thus GSH synthesis or GSSG--reducing enzymes ~~w~~could have been~~as~~ up-regulated. This assumption needs to be verified by determining the~~an~~ accumulation of ROS in the plants harvested under LL conditions and sulfur--deficient plants. Nevertheless, one could assume that the GSH synthesis is regulated by the circadian clock, as there is a CCA1 binding site motif in *GSH1* (http://arabidopsis.med.ohio-state.edu/) catalyzing the first step of the GSH synthesis. The precursor of GSH, cysteine, was measured as well and no diurnal oscillations with a certain period were determined (Fig. 4A). In a study carried out by Huseby et al. (2013) it was demonstrated that the reduced sulfur is first incorporated in GSH and GSLs and at the end of the light phase in proteins thus it is not surprising that the content of cysteine as the source of the reduced sulfur was non-cycling.

Oscillation of GSLs is affected by sulfur limitation

As representatives of the secondary metabolites the individual GSLs were measured in the leaves by HPLC (Fig. 5; Fig. S3, S4). As the sulfur-containing amino acid methionine acts as the precursor for the biosynthesis of aliphatic GSLs those are more sensitive to sulfur deficiency than the indolic and aromatic GSLs (Mailer 1989). In agreement with our results after 4 d of sulfur-deficient conditions only the aliphatic GSL were significantly decreased in their content, whereas the indolic and the aromatic ones were unaffected in their content by the treatment. Regarding the oscillations of the content of the aliphatic, indolic and the one aromatic GSLs ultradian rhythms were shown which were altered under sulfur-deficient conditions. Ultradian rhythms show oscillations shorter than 20 h and were already reported to occur in plants for a number of processes such as glycolysis, sap flow, enzyme activity, root elongation, and leaf movements (Iijima and Matsushita 2011). As the oscillations in the content of all GSLs measured in our study were dependent on the sulfur status the ultradian rhythms might derive from the sulfur supply the plants were grown under. In plants harvested in LL conditions the oscillations in the content of the indolic and aromatic GSLs were maintained under both sulfur regimes indicating that the biosynthesis, degradation or both might be regulated by an endogenous mechanism which can be altered by the sulfur supply. The higher amplitudes of the content of indolic and aromatic GSLs in sulfur deficient plants indicates a stress response directly to sulfur deficient conditions. The biosynthesis and degradation of GSLs in an ultradian rhythm potentially generates breakdown products which could benefit the plant in stress situations. It was shown that allyl isothiocyanate the breakdown product of sinigrin leads to stomatal closure helping water loss in water deficient conditions (Khokon et al. 2011). Furthermore, the breakdown product indole-3-carbinol of the GSL glucobrassicin was shown to antagonize auxin and therefore decelerate growth (Katz et al. 2015). The known functions of

breakdown products in addition to functions that have yet to be elucidated could help the plant to cope with stresses which are the result of low sulfur levels.
The rhythmic oscillations in the content of the aliphatic GSLs were absent under free-running conditions in plants grown under sulfur-deficient conditions. The content of aliphatic GSLs in the sulfur deficient plants was further reduced by LL conditions. It can be assumed that such low levels led to the loss of the rhythmic oscillations in the content of the aliphatic GSLs as the biosynthesis of the aliphatic GSL is more sensitive to sulfur-deficient conditions (Mailer 1989). Regarding the dependence of the oscillations in the content of the GSLs on the sulfur supply, the time of application of sulfur to the plants might also be a factor influencing the oscillations. So far only in the study by Rosa and Rodrigues (1998) ultradian oscillations in the content of GSLs in *B. oleracea* were reported. Diurnal oscillations in the content of the GSLs were already shown in *A. thaliana* (Huseby et al. 2013) where the total GSL content was increased during the day which is contradictory to measurements of the GLSs in *B. oleracea* as they accumulated in the night (Rosa and Rodrigues 1998). These different outcomes were reasoned due to different developmental stages of the plants. Nevertheless, for the GSL content in our study oscillations in the content were comparable to ultradian oscillations, which were highly dependent on the sulfur status.

Conclusions

A growth system for *B. napus* was established to investigate circadian aspects in the sulfur metabolism. In this study the circadian period of the clock in *B. napus* plants entrained to a 12 light/ 12 dark rhythm was 23 h, probably as an adaption to the temperature the plants were grown under. We were able to show diurnal oscillations of genes involved in the transport and reduction of the sulfate with a period comparable to that of *CCA1*. As under free-running conditions the amplitude was lowered and a shift in the phase was determined the circadian control could not be determined unambiguously. The same could be shown for the GSH content measured in the plants. The use of continuous light in this study might have underestimated circadian oscillations as it might have resulted in a stress response affecting the sulfur metabolism in the plants. The GSLs showed ultradian oscillations which were altered by the sulfur supply the plants were grown under. Probably the concentration of single GSLs is not regulated by the circadian clock but in an ultradian way. The analysis of mutants or transgenes in key genes in cysteine and GSH biosynthesis could clarify whether contents of sulfur-containing metabolites are only regulated by the circadian clock because they need reducing equivalents produced in photosynthesis or whether they act as signal molecules.

Material and Methods

Plant material and growth conditions

Seeds from the MSL-hybrid (Male Sterility Lembke) winter oilseed rape cultivar Genie were obtained from the Deutsche Saatveredelung AG (DSV) (Lippstadt, Germany). The cultivar is very vital, has a medium-sized root system and has a high seed oil content in comparison to other cultivars from the DSV. For experiments under circadian and diurnal conditions, the seeds were germinated in a pot (diameter 8 cm) containing sand (0–2 mm grain size, Hornbach, Hannover, Germany) in a climate chamber [22°C, 70% humidity, 12 h light/12 h dark, 480 µmol m^{-2} s^{-1} (lamp type CMT 360LS/W/BH-E40, Eye Lighting Europe Ltd, Uxbridge, UK)]. A total of 102 plants were grown, one plant per pot, for 19 d and watered once per week using 150 ml Blake-Kalff medium (Blake-Kalff et al. 1998) containing 1 mM $MgSO_4$. After a washing step with deionized water one half of the plants was transferred to "plus S" conditions with 1 mM $MgSO_4$. The other half of the plants was transferred to "minus S" conditions using Blake-Kalff medium with 10 µM $MgSO_4$. Plants were grown under these conditions for 4 days. One hour before the light was switched on, shoots of three plants of each treatment were harvested every 4 h over a time period of 36 h. The material was pooled and immediately frozen in liquid nitrogen. Additionally 42 plants under "plus S" and "minus S" conditions were transferred to continuous light. These were then harvested at the same time as the plants grown under 12 h light/12 h dark every 4 h beginning after 16 h representing the beginning of the subjective night. For a 24 h cycle plants under continuous light were additionally harvested at 40 h. The complete experiment was performed twice. Variations of experiment was performed, such the diurnal part or growth on soil instead of sand, were performed several times with the same outcome. Roots of plants were not harvested due to the sand media in which the plants were grown ~~in~~. Fine roots were breaking by removing substrate and analysis of GLS would have been inaccurate due to hydrolysis by myrosinases.

Sequence analysis and primer design

Sequences homologous to *A. thaliana* DNA sequences for *APR2, APR3* and *CCA1* were searched for the primer design in the recently closed *B. napus* database (http://compbio.dfci.harvard.edu/compbio) using BLAST. The data bank used parts of short homologous sequences (high-fidelity virtual transcripts and tentative consensus sequences) to generate EST sequences (Quackenbush et al. 2000). For the primer pair design the program Dosbox with the Primer Design version 2.2 (Scientific & Educational Software, Cary, USA) was used (http://www.dosbox.com). To design the primer pairs for the amplification of cDNA fragments of sulfate transporters, the respective homologous sequences from *Brassica oleracea* were used (Buchner et al. 2004). The amplification of cDNA with the chosen primers generated fragments between 339 and 973 bp (Table 1).

Table 1 Primer pairs used in this study. To identify homologous genes in *B. napus*, the known sequences from *A. thaliana* genes were used to search the *B. napus* database using the BLAST program. s, sense; as, antisense; for, forward; rev, reverse; f, forward; r, reverse. *BoST: BRASSICA*

OLERACEA SULFATE TRANSPORTER; CCA1: CIRCADIAN CLOCK ASSOCIATED1; APR: ADEONOSINE 5'-PHOSPHOSULFATE REDUCTASE; 18S rRNA: 18S RIBOSOMAL RNA; PP2A: SERINE/THREONINE PROTEIN PHOSPHATASE 2A; GDI1: GUANOSINE NUCLEOTIDE DIPHOSPHATE DISSOCIATION INHIBITOR 1.

Primer pairs	***A. thaliana* AGI**	**Sequences**
P216BoST3;1s	At3g51895	5'-TTCTTGTGGCTCGAACACTCCT-3'
P217BoST3;1as		5'-GCCTTACATGTCAACAGCTCTC-3'
P226BoST4;2s	At3g12520	5'-GGTCTTTGACGTGTGAAGCATG-3'
P227BoST4;2as		5'-GTGTACGCTTCTGGATACTGC-3'
P741_Bn_CCA1_for	At2g46830	5'-TTCTTGTGGCTCGAACACTCCT-3'
P742_Bn_CCA1_rev		5'-GGATTGGTGTTGCTGATGACTC-3'
P743_BnAPR2_for	At1g62180	5'-CAAGAAGGAAGATGACACCACC-3'
P744_BnAPR2_rev		5'-GCGAATCGACATCTCTATGCTC-3'
P745_Bn_APR3_for	At4g21990	5'-CATCAAGGAGAACAGCAACGCA-3'
P746_Bn_APR3_rev		5'-TCGGGAACACTAGTATCGTCGG-3'
P782_Bn_18S rRNA_for	X16077.1	5'-ATGAACGAATTCAGACTGTG-3'
P783_Bn_18S rRNA_rev		5'-ACTCATTCCAATTACCAGAC-3'
P968_Bn_PP2A_f	At1g69960	5'-ACGAGGACGGATTTGGTTCC-3'
P969_Bn_PP2A_r		5'-GCTCCGAGCTTGTCATCGAA-3'
P984_Bn_GDI1_f	At2g44100	5'-TGCACGTTTCCAAGGAGGTT-3'
P986_Bn_GDI1_r		5'-CGGTCTGAGGGTTGTCAGTC-3'

Production of probes and Northern blot analysis

Northern blot analysis was performed as described (Weese et al. 2015; Rumlow et al. 2016). Quantitative analysis of the Northern blot results was done by GelAnalyzer5 (http://www.gelanalyzer.com). Normalization of the genes of interests (GOI) was performed with a validated set of reference genes. All aspects for the identification and validation of suitable reference genes (*18S rRNA: 18S RIBOSOMAL RNA; PP2A: SERINE/THREONINE PROTEIN PHOSPHATASE 2A; GDI1: GUANOSINE NUCLEOTIDE DIPHOSPHATE DISSOCIATION INHIBITOR 1*) are described in detail in Rumlow et al. (2016) (Table 1).

Elemental analysis, sulfate determination and extraction and analysis of soluble thiol compounds

Elemental analysis was performed by ICP-OES according to Weese et al. (2015). Sulfate concentrations were analyzed by ICP-AES as described by Bloem et al. (2004). Determination of thiols was done by HPLC after derivatization with monobrombimane according to Riemenschneider et al. (2005).

Analysis of glucosinolates

Samples were prepared as described by Boestfleisch et al. (2017) with some modifications. The content of GSLs was determined using 25 mg freeze-dried material. Glucosinolates were extracted twice with 1 ml 80% (v/v) methanol and centrifuged at 13,000 *g* for 5 min. Before the centrifugation, samples were put on a shaker for 15 min after the first extraction and 30 min after the second extraction at room temperature (RT). The supernatants were pooled and loaded onto a column (Polypropylene column, 1 mL) containing 2 ml of a 5% (w/v) suspension of DEAE Sephadex A25 (Sigma-Aldrich, Taufkirchen, Germany) in 0.5 M acetic acid (pH 5). Columns were washed five times with 2 ml H_2O and two times with 2 ml 0.02 M acetic acid (pH 5). For desulfating the GSLs 50 µl of sulfatase (Sigma-Aldrich) solution was added to 450 µl 0.02 M acetic acid (pH 5) and loaded on to the columns as well (Thies 1979). Desulfation took place over night at RT. Afterwards desulfated GSLs were eluted three times with 2 ml HPLC H_2O and dried overnight in a vacuum centrifuge, and then dissolved in a total amount of 300 µl HPLC H_2O. Analysis was performed with a high-performance-liquid chromatography (HPLC) system (Knauer, Berlin, Germany) equipped with an Ultra AQ C-18 column (150 x 4.6 mm, 5 µm particle size) (Restek GmbH, Bad Homburg, Germany). A water (solvent A)-acetonitrile (solvent B) gradient at a flow rate of 0.5 ml min^{-1} at 45°C (injection volume 50 µl) was as following: The 52 min long run consisted of 100% A for 6 min, 100-70% A for 27 min, 70-40% A for 0.1 min, a 4.9 min hold of 40% A, 40-100 % A for 0.1 min and a 19.9 min hold of 100% A. The detection of the GSL was performed with DAD and FAD (Knauer, Berlin, Germany) at 229 nm. Quantification of the measured GSL was performed by using sinigrin (Phytolab, Vestenbergsgreuth, Germany) as external standard and relative response factors (progoitrin, 1.09; glucoraphanin, 1.07; glucoalyssin, 1.07; gluconapin, 1.11; hydroxyglucobrassicin, 0.17; glucobrassicanapin, 1.15; glucobrassicin, 0.29; gluconasturtiin, 0.95; neoglucobrassicin, 0,2).

Identification of GSLs in *B. napus*

For the identification of the GSL in *B. napus* samples were analyzed by liquid chromatography–mass spectrometry (LC-MS). A volume of 10 µl was injected into the HPLC system (Shimadzu, Darmstadt, Germany) and separated on a Knauer Vertex Plus column (250x 4 mm, 5 µm particle size, packing material ProntoSIL 120-5 C18-H) equipped with a pre-column Knauer, Berlin, Germany). A water (solvent A)-methanol (solvent B), both containing 2 mM ammonium acetate gradient was used with a flow rate of 0.8 ml min^{-1} at 30°C. For measuring the samples, the following gradient was used: 10-90% B for 35 min, 90% for 2 min, 90-10% B for 1 min and 10% B for 2 min. Detection of the spectra in the range 190-800 nm was performed with a diode array detector (SPD-M20A, Shimadzu, Darmstadt, Germany). The HPLC system was coupled to an AB Sciex Triple TOF mass spectrometer (AB Sciex TripleTOF 4600, Canby, USA). At a temperature of 600°C and an ion spray voltage floating of -4500 V the negative electrospray ionization (ESI) was performed. For the ion source gas one and two 50 psi were used and for the curtain gas 35 psi. In the range of 100-1500 Da in the TOF range the mass spectra as well as the MS/MS spectra from 150-1500 Da at a collision energy of -10 eV were recorded. Peaks were identified by analyzing the characteristic mass fragments of ds-progoitrin (195, 309, 344, 617), ds-glucoalyssin (195, 208, 371, 741) and ds-neoglucobrassicin (195, 208, 371, 741). Due to lack of standards of these GSLs fractions of the measured samples were collected in a fraction collector (FRC-10A Shimadzu, Darmstadt, Germany), dried in a vacuum centrifuge and dissolved in 300 µl ultrapure water. The retention time for every GSL was determined by measuring either the collected fraction or the authentic standard (Phytolab, Vestenbergsgreuth, Germany) with the HPLC system as described before.

Databases used for the expression analysis

The database AGRIS (www.arabidopsis.med.ohio-state.edu, Yilmaz et al., 2011) was used to search for circadian clock related binding site motifs. The tool from Mockler et al. (2007) (http://diurnal.mocklerlab.org/) was used to compare array-based transcriptome analysis in *A. thaliana* with the data obtained in this study.

Statistical analysis

The expression levels of GOIs and content levels of sulfur-containing metabolites over time were analyzed using statistical model selection with AICc, a small-sample version of the widely known Akaike Information Criterion (Hurvich and Tsai 1989). Sets of "candidate models" were assembled, and the model with the lowest AICc value was considered to be the one that gets most support from the experimental data (Burnham and Anderson 2002). The "candidates" were linear models with the data for the expression and content of the sulfur-containing metabolites (averages over technical replicates) as dependent variable; the independent variables included light condition, sulfur status and time. Both linear and trigonometric (sine and cosine) functions were considered to model the dependent variable over time. By allowing for interactions between independent variables, the slope and intercept or amplitude, average, and phase shift could or could not depend on light and sulfur. Thereby each of the "candidate models" represented some plausible hypothesis about the underlying biological process e.g. whether gene expression and metabolite content levels are constant or follow a linear trend over time or whether they are governed by a circadian or diurnal periodicity, and if so, what the most likely rhythm is (e.g. 23, 24 or 25 h), and also whether light and sulfur have an impact on expression/content levels and its (periodic) variation over time.
Separate three-way ANOVAs were performed with the values of the expression data and metabolic content as dependent variable and sulfur concentration, time point of harvesting, and light condition (diurnal/circadian) as independent factors as well as their interaction. The factor “light” consisted of two levels: light/light (LL) and light/dark (LD) pattern. Sulfur status consisted of two different concentrations: 0.01 mM $MgSO_4$ and 1 mM $MgSO_4$. The third factor, time, consisted of different harvesting time points, ranging from 0 h to 40 h. Significance of factors and their interactions was assessed by means of F-tests.
Statistical computations were done in R 3.1.1 (RCoreTeam 2014) using add-on package MuMIn (Barton 2014) for the model selection, and in InfoStat version 2016 (http://www.infostat.com.ar) for the ANOVAs. All graphs were generated with SigmaPlot 12.5 (Systat Software, Inc., San Jose, CA).

Author’s contribution

ARi and JP conceived and designed research. JH and ARu conducted experiments. JH, ARu and ~~Ati~~ ARi analyzed data and created the figures. PP and AT performed the statistical analysis. ARu, ARi, JH and JP wrote the manuscript. All authors read and approved the manuscript.

Acknowledgement

We acknowledge the Deutsche Saatveredelung AG, Lippstadt, Germany, for providing us with seeds of the *Brassica napus* cultivar. We would like to thank Julia Volker and Pamela von Trzebiatowski, Hannover, for their excellent technical assistance and the gardener Yvonne Leye for taking care of

the plants. We gratefully acknowledge Dr. Elke Bloem, Julius-Kühn-Institut, Germany, who analyzed the samples for their sulfate content.

References

Barton K (2014) MuMIn: Multi-model inference. R package version 1.12.1. http://CRAN.R-project.org/package=MuMIn

Blake-Kalff MM, Harrison KR, Hawkesford MJ, Zhao FJ, McGrath SP (1998) Distribution of sulfur within oilseed rape leaves in response to sulfur deficiency during vegetative growth. Plant Physiol 118:1337–1344. http://doi.org/10.1104/pp.118.4.1337

BLAST: Basic Local Alignment Search Tool. Available: https://blast.ncbi.nlm.nih.gov/Blast.cgi Accessed 09 November 2016.

Bloem E, Riemenschneider A, Volker J, Papenbrock J, Schmidt A, Salac I, Haneklaus S, Schnug E (2004) Sulphur supply and infection with *Pyrenopeziza brassicae* influence L-cysteine desulphydrase activity in *Brassica napus* L. J Exp Bot 55: 2305–2312. http://doi.org/10.1093/jxb/erh236

Boestfleisch C, Hornbacher J, Rumlow A, Papenbrock J (2017) Contents of single glucosinolates are influenced by salinity in the halophyte *Lepidium latifolium*. In: De Kok L, Hawkesford M, Schnug E (eds) Plant Sulfur Workshop Proceedings, Vol. 3, 2016/17. Springer, Dordrecht. DOI: 10.1007/978-3-319-56526-2_10, pp 103-114.

Brassica napus Genome Browser. Available: http://www.genoscope.cns.fr/brassicanapus/ Accessed 9 November 2016

Buchner P, Stuiver CEE, Westerman S, Wirtz M, Hell R, Hawkesford M, De Kok LJ (2004) Regulation of sulfate uptake and expression of sulfate transporter genes in *Brassica oleracea* as affected by atmospheric H_2S and pedospheric sulfate nutrition. Plant Physiol 136:3396–3408. http://doi.org/10.1104/pp.104.046441

Burnham KP, Anderson DR (2002) Model selection and multimodel inference: a practical information-theoretic approach. Second edition. Springer, New York, NY. doi: 10.1007/b97636

Cao MJ, Wang Z, Wirtz M, Hell R, Oliver DJ, Xiang CB (2013) SULTR3;1 is a chloroplast-localized sulfate transporter in *Arabidopsis thaliana*. Plant J 73:607–616. http://doi.org/10.1111/tpj.12059

CLC Workbench. Available: http://www.clcbio.com/ Accessed 9 November 2016

Computional Biology and Functional Genomics Laboratory: Available http://compbio.dfci.harvard.edu/compbio Accessed 9 November 2016

Covington MF, Maloof JN, Straume M, Kay SA, Harmer SL (2008) Global transcriptome analysis reveals circadian regulation of key pathways in plant growth and development. Genome Biol 9:R130. http://doi.org/10.1186/gb-2008-9-8-r130

Dodd AN, Kusakina J, Hall A, Gould PD, Hanaoka M (2014) The circadian regulation of photosynthesis. Photosynth Res 119:181–190. http://doi.org/10.1007/s11120-013-9811-8

DOSBox. Available: http://www.dosbox.com/ Accessed 9 November 2016

DSV: Deutsche Saatveredelung Available: http://www.dsv-saaten.de/raps/winterraps/sorten/genie.html Accessed: 9 November 2016

Filichkin SA, Breton G, Priest HD, Dharmawardhana P, Jaiswal P, Fox SE, Michael TP, Chory J, Kay SA, Mockler TC (2011) Global profiling of rice and poplar transcriptomes highlights key conserved circadian-controlled pathways and cis-regulatory modules. PloS ONE 6:e16907. http://doi.org/10.1371/journal.pone.0016907

Gelanalyzer: Available: http://gelanalyzer.com/ Accessed 9 November 2016

Gutiérrez RA, Stokes TL, Thum K, Xu X, Obertello M, Katari MS, Tanurdzic M, Dean A, Nero DC, McClung R, Coruzzi GM (2008) Systems approach identifies an organic nitrogen-responsive

gene network that is regulated by the master clock control gene *CCA1*. Proc Natl Acad Sci USA 105:4939–4944. http://doi.org/10.1073/pnas.0800211105

Harmer SL, Hogenesch JB, Straume M, Chang HS, Han B, Zhu T, Wang X, Kreps JA, Kay S (2000) Orchestrated transcription of key pathways in *Arabidopsis* by the circadian clock. Science 290:2110–2113. http://doi.org/10.1126/science.290.5499.2110

Harmer S L (2009) The circadian system in higher plants. Annu Rev Plant Biol 60:357–377. http://doi.org/10.1146/annurev.arplant.043008.092054

Hawkesford MJ, De Kok LJ (2006) Managing sulphur metabolism in plants. Plant Cell Environ 29:382–395. http://doi.org/10.1111/j.1365-3040.2005.01470.x

Haydon, M. J., Bell, L. J., & Webb, A. A. (2011). Interactions between plant circadian clocks and solute transport. J Exp Bot Journal of experimental botany, 62(7): 2333-2348.

Haydon MJ, Román Á, Arshad W (2015) Nutrient homeostasis within the plant circadian network. Front Plant Sci 6:1-6. http://doi.org/10.3389/fpls.2015.00299

Hurvich CM, Tsai CL (1989) Regression and time series model selection in small samples. Biometrika 76:297-307.

Huseby S, Koprivova A, Lee BR, Saha S, Mithen R, Wold AB, Bengtsson GB, Kopriva S (2013) Diurnal and light regulation of sulphur assimilation and glucosinolate biosynthesis in *Arabidopsis*. J Exp Bot 64:1039–48. http://doi.org/10.1093/jxb/ers378

Iijima M, Matsushita N (2011) A circadian and an ultradian rhythm are both evident in root growth of rice. J Plant Physiol 168:2072–2080. doi:10.1016/j.jplph.2011.06.005

InfoStat. Available: http://www.infostat.com.ar/?lang=en Accessed 9 November 2016

Kataoka T, Watanabe-Takahashi A, Hayashi N, Ohnishi M, Mimura T, Buchner P, Hawkesford MJ, Yamaya T, Takahashi H (2004)Vacuolar sulfate transporters are essential determinants controlling internal distribution of sulfate in *Arabidopsis*. Plant Cell 16:2693–2704. doi:10. 1105/tpc. 104. 023960

Katz E, Nisani S, Yadav BS, Woldemariam MG, Shai B, Obolski U, Chamovitz DA (2015) The glucosinolate breakdown product indole-3-carbinol acts as an auxin antagonist in roots of *Arabidopsis thaliana*. The Plant Journal 82:547-555.

Kerwin RE, Jimenez-Gomez JM, Fulop D, Harmer SL, Maloof JN, Kliebenstein DJ (2011) Network quantitative trait loci mapping of circadian clock outputs identifies metabolic pathway-to-clock linkages in *Arabidopsis*. The Plant Cell 23:471–485. http://doi.org/10.1105/tpc.110.082065

Khokon MAR, Jahan MS, Rahman T, Hossain MA, Muroyama D, Minami I, Murata Y (2011) Allyl isothiocyanate (AITC) induces stomatal closure in *Arabidopsis*. Plant Cell Environ 34:1900-1906.

Kopriva S, Muheim R, Koprivova A, Trachsel N, Catalano C, Suter M, Brunold C (1999) Light regulation of assimilatory sulphate reduction in *Arabidopsis thaliana*. Plant J 20:37–44. http://doi.org/10.1046/j.1365-313X.1999.00573.x

Kusakina J, Gould PD, and Hall A (2014) A fast circadian clock at high temperatures is a conserved feature across *Arabidopsis* accessions and likely to be important for vegetative yield. Plant Cell Environ 37(2), 327-340

Lai AG, Doherty CJ, Mueller-Roeber B, Kay SA, Schippers JHM, Dijkwel PP (2012) *CIRCADIAN CLOCK-ASSOCIATED 1* regulates ROS homeostasis and oxidative stress responses. Proc Natl Acad Sci USA 109:17129–17134. http://doi.org/10.1073/pnas.1209148109

Mailer RJ (1989) Effects of applied sulfur on glucosinolate and oil concentrations in the seeds of rape *Brassica napus* L. and turnip rape *Brassica rapa* L. var. *silvestris* (Lam.) Briggs. Aust J Agric Res 40:617–624.

McClung CR (2006) Plant circadian rhythms. Plant Cell 18:792–803. http://doi.org/10.1017/CBO9781107415324.004

Formatted: Font: (Default) +Body (Calibri), 11 pt, Not Italic, Font color: Auto, English (United States)

Formatted: Font: (Default) +Body (Calibri), 11 pt, Not Italic, Font color: Auto, English (United States)

Mithöfer A, Boland W (2012) Plant defense against herbivores: chemical aspects. Annu Rev Plant Biol 63:431-450. doi: 10.1146/annurev-arplant-042110-103854.

Mizoguchi T, Wheatley K, Hanzawa Y, Wright L, Mizoguchi M, Song HR, Carré IA, Coupland G (2002) *LHY* and *CCA1* are partially redundant genes required to maintain circadian rhythms in *Arabidopsis*. Dev Cell 2:629–641. doi:10.1016/S1534-5807(02)00170-3

Mockler TC, Michael TP, Priest HD, Shen R, Sullivan CM, Givan SA, McEntee C, Kay SA, Chory J (2007) The diurnal project: diurnal and circadian expression profiling, model-based pattern matching, and promoter analysis. Cold Spring Harb Symp Quant Biol 72:353–363. doi:10.1101/sqb.2007.72.006

Nagel DH, Doherty CJ, Pruneda-Paz J L, Schmitz RJ, Ecker JR, Kay SA (2015) Genome-wide identification of CCA1 targets uncovers an expanded clock network in *Arabidopsis*. Proc Natl Acad Sci USA 112:E4802–4810. http://doi.org/10.1073/pnas.1513609112

Noctor G, Arisi ACM, Jouanin L, Valadier MH, Roux Y, Foyer CH (1997) Light-dependent modulation of foliar glutathione synthesis and associated amino acid metabolism in poplar overexpressing γ-glutamylcysteine synthetase. Planta 202:357–369. http://doi.org/10.1007/s004250050138

Parmar S, Buchner P, Hawkesford MJ (2007) Leaf developmental stage affects sulfate depletion and specific sulfate transporter expression during sulfur deprivation in *Brassica napus* L. Plant Biol. 9:647–653. http://doi.org/10.1055/s-2007-965428

Quackenbush J, Liang F, Holt I, Pertea G, Upton J (2000) The TIGR gene indices: reconstruction and representation of expressed gene sequences. Nucleic Acids Res 28:141–145.

R Core Team (2014). *R: A Language and Environment for Statistical Computing.* Vienna: R Foundation for Statistical Computing. Available online at: http:// www.R-project.org

Reich M, Aghajanzadeh T, Helm J, Parmar S, Hawkesford MJ, De Kok LJ (2017) Chloride and sulfate salinity differently affect biomass, mineral nutrient composition and expression of sulfate transport and assimilation genes in *Brassica rapa*. Plant Soil, 411:319–332. doi:10.1007/s11104-016-3026-7

Riemenschneider A, Nikiforova V, Hoefgen R, De Kok LJ, Papenbrock J (2005) Impact of elevated H_2S on metabolite levels, activity of enzymes and expression of genes involved in cysteine metabolism. Plant Physiol Biochem 43:473-483.

RobiNA. Available: http://www.bioinformatics.babraham.ac.uk/projects/fastqc/ Accessed 9 November 2016

Rosa AS, Rodrigues PMF (1998)The effect of light and temperature on glucosinolate concentration in the leaves and roots of cabbage seedlings. J Sci Food Agric 78:208–212.

Rumlow A, Keunen E, Klein J, Pallmann P, Riemenschneider A, Cuypers A, Papenbrock J (2016) Quantitative expression analysis in *Brassica napus* by Northern blot analysis and reverse transcription-quantitative PCR in a complex experimental setting. PLoS ONE 11:e0163679.doi:10.1371/journal.pone.0163679

Salomé PA, McClung CR (2004) The *Arabidopsis thaliana* clock. J Biol Rhythms 19:425–435. doi:10.1177/0748730404268112

Schnug E, Haneklaus S (2005) Sulphur deficiency symptoms in oilseed rape (*Brassica napus* L.) - the aesthetics of starvation. Phyton 45:79-95.

Scherer HW (2001) Sulphur in crop production — invited paper. Eur J Agron 14:81–111. doi:10.1016/S1161-0301(00)00082-4

Shaw LM, Turner AS, Laurie DA (2012) The impact of photoperiod insensitive *Ppd-1a* mutations on the photoperiod pathway across the three genomes of hexaploid wheat (*Triticum aestivum*). Plant J 71:71–84. doi:10.1111/j.1365-313X.2012.04971.x

Thies W (1979) Detection and utilization of a glucosinolate sulfohydrolase in the edible snail, *Helix pomatia*. Naturwissenschaften 66:364-365.

Vauclare P, Kopriva S, Fell D, Suter M, Sticher L, von Ballmoos P, Krähenbühl U, den Camp RO, Brunold C (2002) Flux control of sulphate assimilation in *Arabidopsis thaliana* : adenosine 5'-phosphosulphate reductase is more susceptible than ATP sulphurylase to negative control by thiols. Plant J 31:729–740. doi:10.1046/j.1365-313X.2002.01391.x

Velez-Ramirez AI, van Ieperen W, Vreugdenhil D, Millenaar FF (2011) Plants under continuous light. Trends Plant Sci 16:310–318. doi:10.1016/j.tplants.2011.02.003

Wang W, Barnaby JY, Tada Y, Li H, Tör M, Caldelari D, Lee D, Fu XD, Dong X (2011) Timing of plant immune responses by a central circadian regulator. Nature 470:110–114. doi:10.1038/nature09766.

Weese A, Pallmann P, Papenbrock J, Riemenschneider A (2015) *Brassica napus* L. cultivars show a broad variability in their morphology, physiology and metabolite levels in response to sulfur limitations and to pathogen attack. Front Plant Sci 6. doi:10.3389/fpls.2015.00009

Yilmaz A, Mejia-Guerra MK, Kurz K, Liang X, Welch L, Grotewold E (2011) AGRIS: the *Arabidopsis* gene regulatory information server, an update. Nucleic Acids Res 39:D1118-D1122

Supporting information

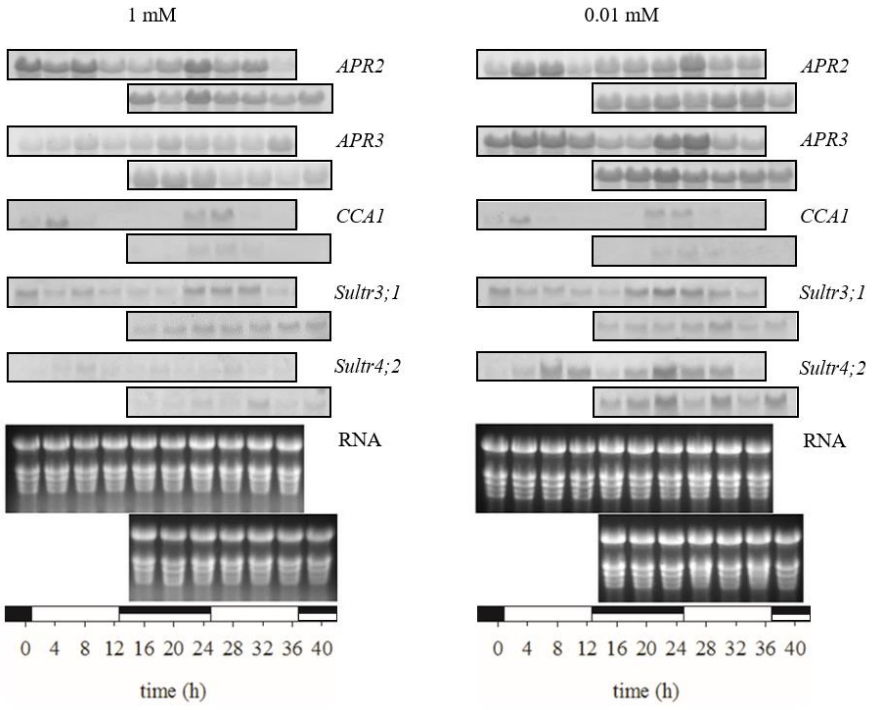

Fig. S1 Northern blot analysis of the GOIs under diurnal/circadian conditions. Transcript amounts were determined in above ground plant material of plants (with five fully expanded leaves) grown using 1 mM $MgSO_4$ as a control and 0.01 mM $MgSO_4$ for four days to obtain S-deficient conditions. Plants grown under 12 h dark/ 12 h light (LD) were harvested over a period of 36 h every 4 h starting 1 h before the onset of light (first row). In addition, plants grown in a chamber with 24 h light (LL) were parallel harvested beginning at 16 h (second row). Total RNA was isolated, and for Northern blot analysis 15 µg RNA was electrophoretically separated and transferred onto membranes. For the detection DIG labeled probes were used. Results for one technical replicate are shown. For abbreviations see Table 1.

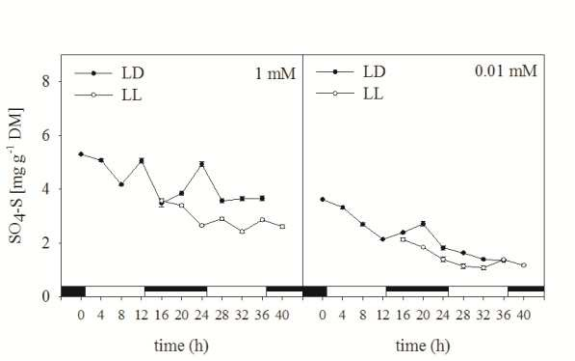

Fig. S2 The content of sulfate-sulfur in the leaves under diurnal/circadian conditions. The sulfate content in the above ground plant material treated and harvested as described for Figure 2 was determined in 500 mg freeze dried material by ion chromatography. Calculated data for the sulfate-sulfur in mg g^{-1} DW represent two technical replicates ±SD.

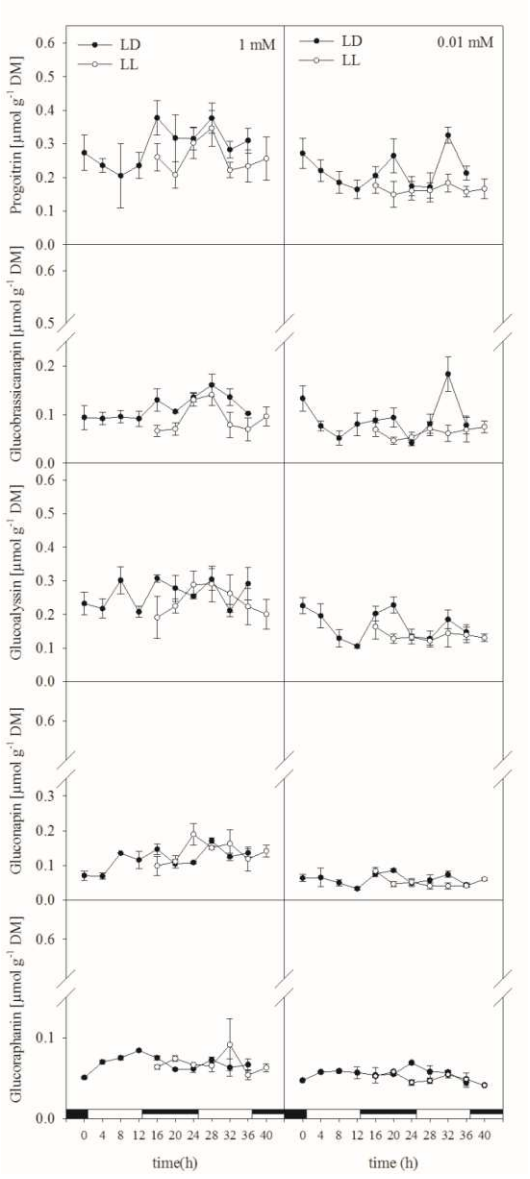

Fig. S3 Individual aliphatic GSL in leaves under diurnal/circadian conditions. Individual aliphatic GSL were identified by LC-MS and quantified by HPLC measurements in above ground plant material of plants treated and collected as described in Figure 2. The contents of the GSLs were calculated in µmol g^{-1} FM. Data represent the mean of three technical replicates ±SD.

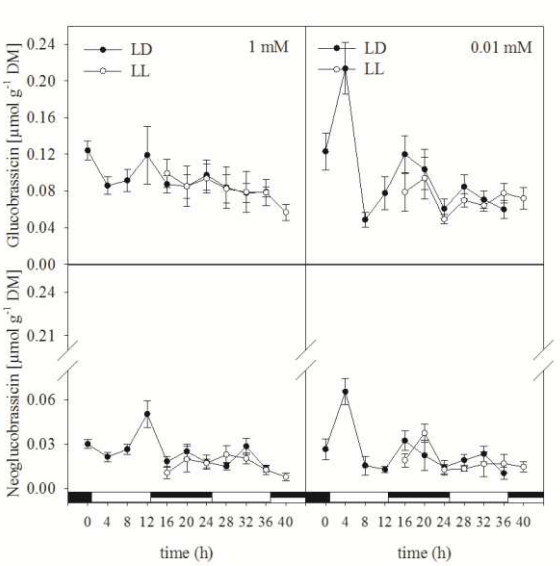

Fig. S4 Individual indolic GSL in leaves under diurnal/circadian conditions. Individual indolic GSL were identified by LC-MS and quantified by HPLC measurements in above ground plant material of plants treated and collected as described in Figure 2. The contents of the GSLs were calculated in µmol g^{-1} FM. Data represent the mean of three technical replicates ±SD.

Table S1 Three-way ANOVA analysis of the expression data and the measured sulfur-containing compounds under diurnal and circadian conditions based on three technical replicates.

Sulfur status (S)	**S -**												**S +**												***p*-values**						
Time point of harvest (T)	**16**	**16**	**20**	**20**	**24**	**24**	**28**	**28**	**32**	**32**	**36**	**36**	**16**	**16**	**20**	**20**	**24**	**24**	**28**	**28**	**32**	**32**	**36**	**36**							
Light (L)	**LD**	**LL**	**LD**	**LL**	**LD**	**LL**	**LD**	**LL**	**LD**	**LL**	**LD**	**LL**	**LD**	**LL**	**LD**	**LL**	**LD**	**LL**	**LD**	**LL**	**LD**	**LL**	**LD**	**LL**	**S**	**T**	**L**	**SxL**	**SxT**	**LxT**	**SxLxT**
APR_2 (rel. expression)	0.02	0.03	0.02	0.04	0.05	0.04	0.05	0.04	0.03	0.03	0.02	0.04	0.01	0.03	0.02	0.03	0.04	0.04	0.03	0.01	0.02	0.02	0.02	0.02	<0.0001	<0.0001	0.0561	0.2427	0.0135	0.0007	0.2067
APR_3 (rel. expression)	0.03	0.04	0.02	0.05	0.05	0.05	0.05	0.04	0.02	0.04	0.02	0.05	0.02	0.02	0.03	0.02	0.02	0.03	0.01	0.02	0.02	0.02	0.02	0.02	<0.0001	0.0217	0.0072	0.0119	0.0646	0.2059	0.0141
CCA1 (rel. expression)	0.01	0.01	4.70E-03	4.90E-03	0.08	0.04	0.11	0.05	0.04	0.03	0.01	0.02	0.01	3.00E-03	0.01	0.01	0.09	0.03	0.06	0.04	0.02	0.02	0.01	0.01	<0.0001	<0.0001	<0.0001	0.0291	<0.0001	<0.0001	<0.0001
Sultr3;1 (rel. expression)	0.02	0.02	0.04	0.02	0.05	0.03	0.05	0.03	0.04	0.03	0.02	0.03	0.02	0.02	0.02	0.02	0.05	0.02	0.04	0.02	0.04	0.03	0.02	0.03	0.0723	<0.0001	<0.0001	0.6544	0.7347	<0.0001	0.3965
Sultr4;2 (rel. expression)	0.03	0.04	0.05	0.04	0.07	0.06	0.07	0.05	0.05	0.06	0.03	0.05	0.01	0.02	0.01	0.02	0.01	0.02	0.01	0.01	0.01	0.02	0.02	0.01	<0.0001	0.0183	0.2711	0.7722	0.0741	0.1772	0.1239
Total sulfur (mg g-1 DM)	4.74	4.66	4.6	3.54	4.34	3.65	4.38	3.65	4.12	3.09	4.14	2.74	5.57	5.45	6.86	6.44	5.36	5.8	6.01	5.17	6.56	5.46	6.27	5.38	<0.0001	0.0312	<0.0001	0.185	0.003	0.0938	0.6654
Sulfate (mg g-1 DM)	2.39	2.12	2.71	1.84	1.82	1.39	1.63	1.14	1.39	1.08	1.34	1.38	3.49	3.56	3.84	3.39	4.93	2.65	3.56	2.9	3.65	2.42	3.66	2.86	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
GSH (nmol g-1 FM)	342.9	382.81	271.04	361.59	285.03	368.7	322.23	366.16	403.08	344.65	372.94	334.35	308.22	448.24	292.99	412.04	240.25	401.55	376.26	452.36	559.35	462.41	443.07	404.02	0.0002	0.0002	0.0018	0.191	0.0396	0.0003	0.6312
Cystein (nmol g-1 FM)	11.31	9.86	11.08	7.87	11.19	8.35	10.05	9	10.41	9.69	9.86	10.39	11.82	12.18	11.93	11.9	12.43	14.03	16.06	12.2	20.64	15.95	13.36	14.4	0.0001	0.3206	0.1863	0.7663	0.335	0.8574	0.7001
Aliphatic GSLs (µmol g-1 DM)	0.62	0.54	0.72	0.44	0.47	0.44	0.5	0.44	0.82	0.48	0.52	0.44	1.02	0.68	0.86	0.69	0.87	0.99	1.08	0.99	0.81	0.78	0.91	0.69	<0.0001	0.1421	<0.0001	0.649	0.0001	0.0212	0.0153
Indolic GSLs (µmol g-1 DM)	0.15	0.09	0.12	0.14	0.07	0.06	0.1	0.08	0.09	0.08	0.07	0.09	0.1	0.11	0.11	0.1	0.11	0.11	0.1	0.1	0.1	0.09	0.09	0.09	0.3288	0.0066	0.1931	0.4712	0.0209	0.4621	0.2137
Progoitrin (µmol g-1 DM)	0.2	0.18	0.26	0.15	0.18	0.16	0.17	0.16	0.3	0.18	0.25	0.16	0.38	0.18	0.32	0.21	0.32	0.3	0.38	0.35	0.28	0.31	0.31	0.21	<0.0001	0.2401	<0.0001	0.6282	0.0015	0.0321	0.0087
Glucoraphanin (µmol g-1 DM)	0.05	0.05	0.06	0.06	0.06	0.04	0.06	0.05	0.06	0.05	0.05	0.05	0.07	0.06	0.06	0.07	0.06	0.07	0.07	0.07	0.06	0.09	0.07	0.05	0.0007	0.4794	0.5492	0.2016	0.9431	0.4455	0.4128
Glucoalyssin (µmol g-1 DM)	0.18	0.16	0.23	0.13	0.14	0.13	0.13	0.12	0.18	0.14	0.16	0.14	0.39	0.18	0.28	0.27	0.3	0.31	0.4	0.31	0.26	0.33	0.29	0.22	<0.0001	0.6481	0.0015	0.4506	0.0196	0.0436	0.0065
Gluconapin (µmol g-1 DM)	0.05	0.06	0.08	0.05	0.06	0.05	0.05	0.04	0.07	0.04	0.06	0.04	0.15	0.1	0.1	0.11	0.11	0.19	0.17	0.15	0.13	0.16	0.14	0.12	<0.0001	0.567	0.5549	0.1429	0.1434	0.2888	0.0608
Glucobrassicanapin (µmol g-1 DM)	0.09	0.07	0.09	0.05	0.05	0.05	0.08	0.07	0.17	0.06	0.09	0.07	0.12	0.07	0.12	0.07	0.13	0.13	0.15	0.14	0.13	0.08	0.09	0.07	<0.0001	0.0013	<0.0001	0.647	<0.0001	0.0001	0.1741
Glucobrassicin (µmol g-1 DM)	0.11	0.08	0.11	0.09	0.06	0.05	0.08	0.07	0.08	0.06	0.06	0.08	0.09	0.07	0.08	0.09	0.1	0.09	0.08	0.08	0.08	0.09	0.08	0.08	0.0757	0.0298	0.1026	0.167	0.0004	0.2213	0.6741
Gluconasturtiin (µmol g-1 DM)	0.07	0.05	0.08	0.06	0.06	0.03	0.05	0.04	0.05	0.05	0.04	0.04	0.07	0.05	0.06	0.05	0.07	0.07	0.06	0.05	0.06	0.05	0.05	0.04	0.4192	0.0082	0.0007	0.5925	0.0129	0.6681	0.7074
Neoglucobrassicin (µmol g-1 DM)	0.03	0.02	0.03	0.04	0.02	0.01	0.02	0.01	0.02	0.02	0.01	0.02	0.02	0.02	0.03	0.02	0.02	0.02	0.02	0.02	0.03	0.03	0.01	0.02	0.311	0.0001	0.412	0.9839	0.0487	0.8406	0.1761